Pergamon

PII: S0042-6989(96)00034-X

Vision Res., Vol. 36, No. 18, pp. 2797–2804, 1996

Printed in Great Britain
0042-6989/96 $15.00 + 0.00

Phylogenetic Relationships among Short Wavelength-sensitive Opsins of American Chameleon (*Anolis carolinensis*) and Other Vertebrates

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Received 11 July 1995; in revised form 14 November 1995; in final form 22 January 1996

The vertebrate opsins have been classified into four major phylogenetic groups. One of them, a short wavelength-sensitive (SWS) opsin group, is further divided into two subgroups, SWS-I and SWS-II, having the wavelengths of maximal absorption of about 420 and 450 nm, respectively. Here we report the DNA sequences of the SWS-I and SWS-II genes from the lizard *Anolis carolinensis*. The shorter wavelengths of absorption by the two SWS subgroup opsins seem to be achieved by different sets of amino acid replacements in the transmembrane regions. Copyright © 1996 Elsevier Science Ltd.

Opsin genes Molecular evolution Vertebrates Color vision

INTRODUCTION

In humans, visual pigments in rods mediate vision in dim light and have a wavelength of maximal absorption (λ_{max}) of 495 nm, while those in the three types of cones are responsible for color vision, having λ_{max} values of 420 nm (short wavelength-sensitive: SWS), 530 nm (middle wavelength-sensitive: MWS), and 560 nm (long wavelength-sensitive: LWS) (Bowmaker, 1991). Each of these visual pigments consists of a chromophore, 11-*cis* retinal (vitamin A1 aldehyde), and a transmembrane (TM) protein, opsin, which is encoded by one of the four distinct opsin genes (Nathans & Hogness, 1984; Nathans *et al.*, 1986). The human visual system exemplifies the basic features of vertebrate vision. That is, only a few types of visual pigments are needed for animals to visualize a wide range of the spectrum.

Vertebrate opsins may be classified into RH1, RH2, LWS/MWS, and SWS opsins (Yokoyama, 1994). The SWS group may further be distinguished into two subgroups: SWS-I and SWS-II [see also Okano *et al.* (1992) and Hisatomi *et al.* (1994) for the classification of opsins]. When currently known SWS-I, SWS-II, RH1, RH2, and LWS/MWS opsins are reconstituted with 11-*cis* retinal, the visual pigments produced attain λ_{max} values of about 420, 450, 500, 470–510, and 520–570 nm, respectively (Yokoyama, 1995). Surprisingly, the ultraviolet-sensitive (UV) opsin of zebrafish (Robinson *et al.*, 1993), associated with a λ_{max} of 360 nm, belongs to the RH1 group (Yokoyama & Yokoyama, 1993; Yokoyama, 1995).

The American chameleon, *Anolis carolinensis*, a diurnal and arboreal reptile, has a unique visual system, possessing an all-cone retina and only 11-*cis*-3,4-dehydroretinal (vitamin A2 aldehyde) as a chromophore (Provencio *et al.*, 1992). Vitamin A2-based visual pigments (A2-pigments) usually absorb at higher wavelengths than vitamin A1-based visual pigments (A1-pigments) (e.g. see Whitmore & Bowmaker, 1989). The three distinct types of cones in this species have λ_{max} of 462, 503, and 625 nm, with the last value being some 50 nm further into the red than any other terrestrial vertebrates examined to date (Provencio *et al.*, 1992). So far, one gene each encoding RH1 (Kawamura & Yokoyama, 1994), RH2 (Kawamura & Yokoyama, 1995), and LWS/MWS (Kawamura & Yokoyama, 1993) opsins of the American chameleon have been characterized, where the RH1 gene is suspected to be expressed exclusively in the pineal gland (see Foster *et al.*, 1993).

Here we report the remaining two SWS opsin genes from the American chameleon genome. The number of opsin genes characterized in the American chameleon genome is larger than that of the three λ_{max} values identified. Interestingly, closely related *Anolis* species which live in Puerto Rico have UV-vision (Fleishman *et al.*, 1993) and, therefore, the extra opsin gene in the

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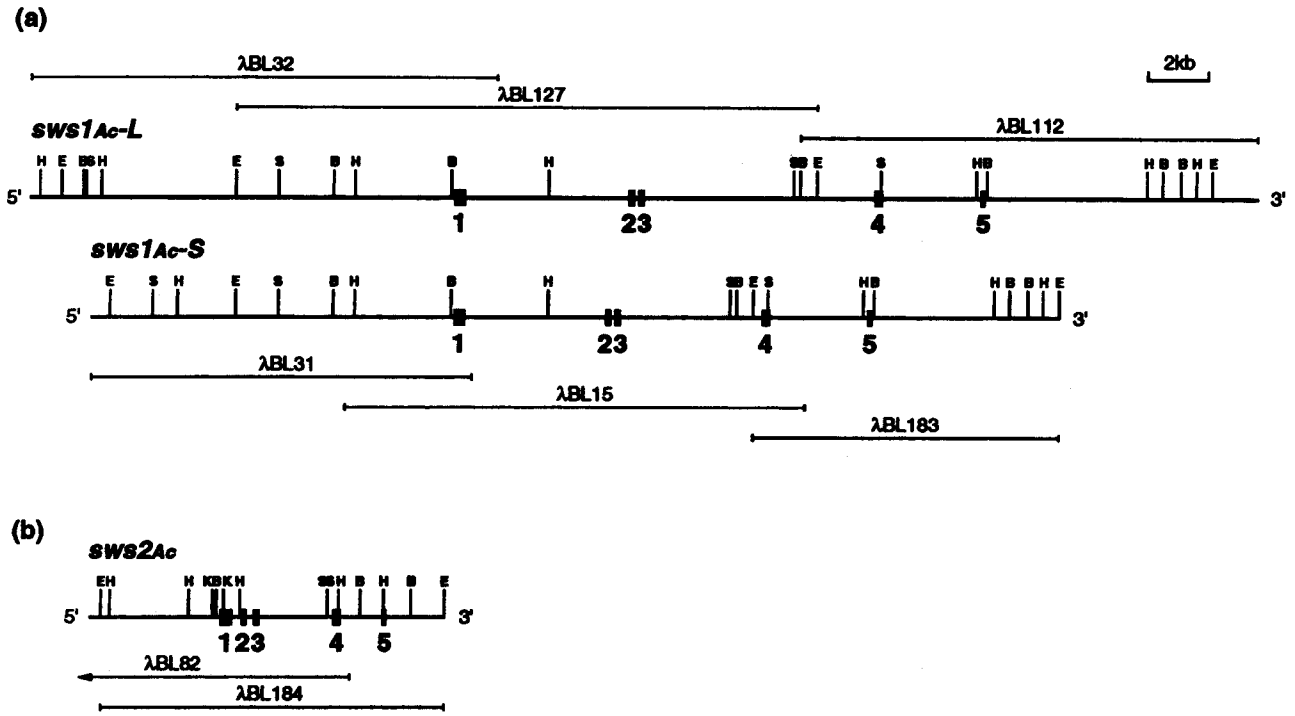

FIGURE 1. Restriction maps of (a) the two SWS-I genes ($sws1_{Ac}$-*L* and $sws1_{Ac}$-*S*), and (b) the SWS-II gene ($sws2_{Ac}$) of the American chameleon. The five coding exons are indicated by solid boxes in the restriction maps. Horizontal bars above and below the maps represent λ clones. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Sst*I.

American chameleon may be closely related to the gene encoding the UV opsins in the Puerto Rican *Anolis* species. So far, SWS genes have been isolated from human (Nathans *et al.*, 1986), Old and New World monkeys (Hunt *et al.*, 1995), mouse (Chiu *et al.*, 1994), bovine (Chiu *et al.*, 1994), chicken (Okano *et al.*, 1992), goldfish (Johnson *et al.*, 1993), and cavefish, *Astyanax fasciatus* (Yokoyama & Yokoyama, 1993). Thus, the present study provides the first reptilian SWS genes and significantly improves the variety of vertebrate species from which SWS genes are sampled, making the evolutionary analyses of the opsin genes more general.

METHODS

Genomic library screening and DNA sequencing

A genomic library was constructed by using the high molecular weight DNA made from one American chameleon (Kawamura & Yokoyama, 1993). We screened 1.8×10^6 recombinant plaques for the SWS-I and SWS-II genes using the full-length cDNA for the human SWS gene [hs37 in Nathans *et al.* (1986); kindly provided by Dr J. Nathans] and PCR-amplified exons 1 and 4 of the cavefish AF23 (Yokoyama & Yokoyama, 1993) as probes, respectively. Note that the human and fish genes are shown to belong to the SWS-I and SWS-II groups, respectively (Yokoyama, 1994). Labeling, plaque hybridization, and membrane washing were performed as previously described (Kawamura & Yokoyama, 1993). We obtained two sets of SWS-I clones [λBL32 and λBL112 for $sws1_{Ac}$-*L* and λBL31 and λBL15 for $sws1_{Ac}$-*S*; Fig. 1(a)] and one SWS-II clone [λBL82 for $sws2_{Ac}$; Fig. 1(b)] after three rounds of plaque hybridizations. Since they did not contain the entire coding regions [Fig. 1(a) and (b)], we further screened the *Eco*RI-library prepared in a previous study (Kawamura & Yokoyama, 1995) and obtained λBL127, λBL183, and λBL184 [Fig. 1(a) and (b)]. Linkages of the three sets of λ clones were determined using their restriction maps and partial nucleotide sequences. Subcloning and nucleotide sequencing for both strands were performed as previously described (Kawamura & Yokoyama, 1993).

Southern blot analysis

Restriction digestion and Southern blotting of the American chameleon genomic DNA were performed as previously described (Kawamura & Yokoyama, 1993). Exons 4 of $sws2_{Ac}$ and the human SWS gene were PCR amplified from λBL82 and hs37, respectively (each 240 bp long) and used sequentially for the same blot as hybridization probes. Labeling and hybridization were done as previously described (Kawamura & Yokoyama, 1993). The hybridized membrane was washed four times (30 min each) in 1×SSC (0.15 M NaCl/0.015 M Na_3·citrate)/0.1% SDS at 55°C for low stringency condition and at 65°C for moderate stringency condition, which allows ~30% and ~20% mismatches, respectively (Sambrook *et al.*, 1989). Before re-hybridization, the old probe was removed from the blot by washing it in

0.4 M NaOH at 45°C for 30 min and then in 0.1 × SSC/0.1% SDS/0.2 M Tris (pH 7.5) at 45°C for 30 min.

Sequence analysis

The amino acid sequences deduced from *sws1*$_{Ac}$*-L* and *sws1*$_{Ac}$*-S* turn out to be identical and are denoted as SWS-I, whereas that deduced from *sws2*$_{Ac}$ is denoted as SWS-II. The RH1, RH2, LWS, and SWS-I and SWS-II opsins of the American chameleon were compared with the corresponding opsins (rhodopsin, green, red, blue, violet opsins) of chicken [*Gallus gallus*; Okano *et al.* (1992)]. To construct a rooted phylogenetic tree for these ten opsins, we used invertebrate opsins from *D. melanogaster* (GenBank K02315), octopus (*Paroctupus defleini*; X07797), and squid (*Loligo forbesi*; X56788).

SWS opsins of the American chameleon were further compared to those from other vertebrates: human [*Homo sapiens*; Nathans *et al.* (1986)], bovine [*Bos taurus*; Chiu *et al.* (1994)], mouse [*Mus musculus*; Chiu *et al.* (1994)], chicken [*Gallus gallus*; Okano *et al.* (1992)], goldfish [*Carassius auratus*; Johnson *et al.* (1993)], and cavefish [*Astyanax fasciatus*; Yokoyama & Yokoyama (1993)]. To construct a rooted phylogenetic tree for these nine SWS opsins, we used the RH1 opsins from the brook lamprey (GenBank; M63632), *X. laevis* (L07770), chicken (D00702), human (K02281), and bovine (K00502-K00506) as outgroups.

After aligning these sequences, the number (K) of amino acid substitutions per site for two sequences was estimated from $K = -\ln(1 - p - p^2/5)$, where p is the proportion of different amino acids between the two sequences (Kimura, 1983). We estimated the topology and branch lengths of the phylogenetic tree by using the neighbor-joining (NJ) method (Saitou & Nei, 1987) based on the K values. The reliability of the NJ tree topology was evaluated by the bootstrap analysis with 1000 replications [CLUSTAL V; Higgins *et al.* (1992)].

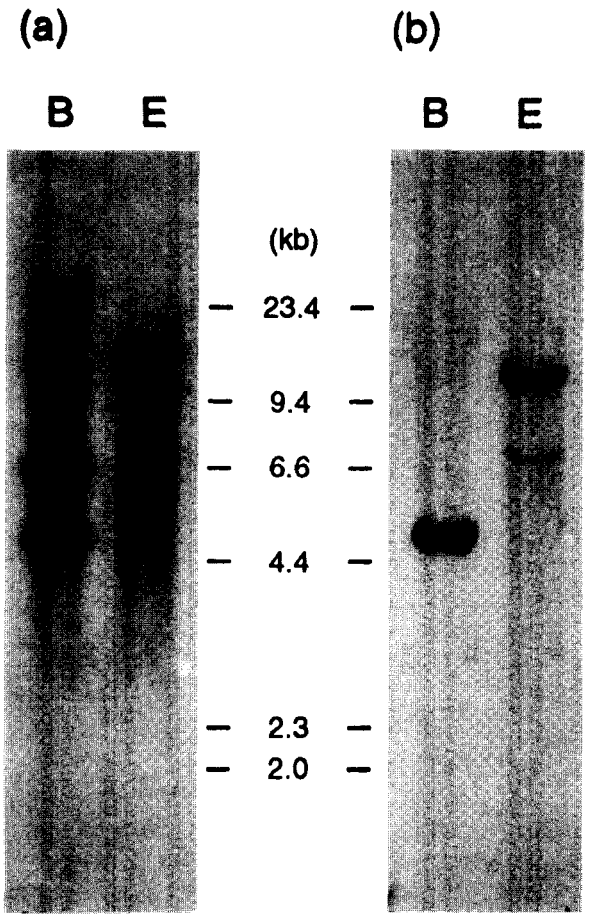

FIGURE 2. Southern hybridizations of the genomic DNA of the American chameleon with exon 4 of (a) the human SWS gene and (b) that of *sws2*$_{Ac}$ under moderately stringent washing conditions (see Methods Section 2). The B and E lanes correspond to *Bam*HI and *Eco*RI digests, respectively. λ *Hin*dIII size standards are indicated in kb.

RESULTS AND DISCUSSION

The SWS genes of the American chameleon

The two SWS-I genes, *sws1*$_{Ac}$*-L* and *sws1*$_{Ac}$*-S*, contain five putative exons and four introns and span about 17.4 kb and 13.8 kb from the start codon to the stop codon, respectively [Fig. 1(a)]. The length difference between the two genes is due to *sws1*$_{Ac}$*-L* having an extra 752 bp in intron 1 and a total of 2.9 kb extra in intron 3. The 3′-flanking region of *sws1*$_{Ac}$*-L* contains 1.3 kb more than that of *sws1*$_{Ac}$*-S* before identical restriction enzyme maps resume [Fig. 1(a)]. Furthermore, the restriction maps of the two genes differ in the region 8 kb upstream from exon 1 [Fig. 1(a)].

When exon 4 of the human SWS gene, belonging to the SWS-I group, is hybridized to the genomic Southern blot under moderately stringent conditions (see Methods), two bands in each of the two restriction digests are detected [Fig. 2(a)], the sizes of which exactly correspond to the cloned genes. When exon 1 of the human SWS gene is used as a probe, the banding pattern is again consistent with the restriction maps of the clones (result not shown). Using the low stringency condition (see Methods), additional bands corresponding to SWS-II (see below), RH1 and RH2 genes can be detected (result not shown), strongly suggesting that *sws1*$_{Ac}$*-L* and *sws1*$_{Ac}$*-S* are the only SWS-I genes in the American chameleon genome.

Despite their structural differences, the coding nucleotide sequences of *sws1*$_{Ac}$*-L* and *sws1*$_{Ac}$*-S* are virtually identical, showing only one silent nucleotide change in exon 1 [Fig. 3(a)]. In intron regions sequenced (entire regions of introns 2 and 4 and portions of introns 1 and 3), there are 32 nucleotide substitutions at the 5111 bp sites compared. From this proportion of different nucleotides, the number of nucleotide substitutions per site is estimated to be 0.0063. Assuming that the intron regions of the two SWS-I opsin genes are evolving at the same rates as those of mammalian genes, i.e. about 3.7×10^{-9}/site/yr (Li *et al.*, 1985), the divergence time between *sws1*$_{Ac}$*-L* and *sws1*$_{Ac}$*-S* is estimated to be 0.85 million yr ago. The recent origin of *sws1*$_{Ac}$*-L* and *sws1*$_{Ac}$*-S* suggests that they are most likely to be different alleles rather than

FIGURE 3. The nucleotide and the deduced amino acid sequences of *sws1*$_{Ac}$*-L* and *sws1*$_{Ac}$*-S* (a) and those of *sws2*$_{Ac}$ (b). (a) A different nucleotide of *sws1*$_{Ac}$*-S* from *sws1*$_{Ac}$*-L* is indicated below the nucleotide sequence of *sws1*$_{Ac}$*-L*. Introns 1, 2, 3, and 4 consist of 5.4 kb, 144 bp, 7.6 kb, and 3232 bp, respectively, in *sws1*$_{Ac}$*-L*, and 4.7 kb, 144 bp, 4.7 kb, and 3232 bp, respectively, in *sws1*$_{Ac}$*-S*. (b) Lengths of introns 1, 2, 3, and 4 are 300, 271, 2414, and 1384 bp, respectively.

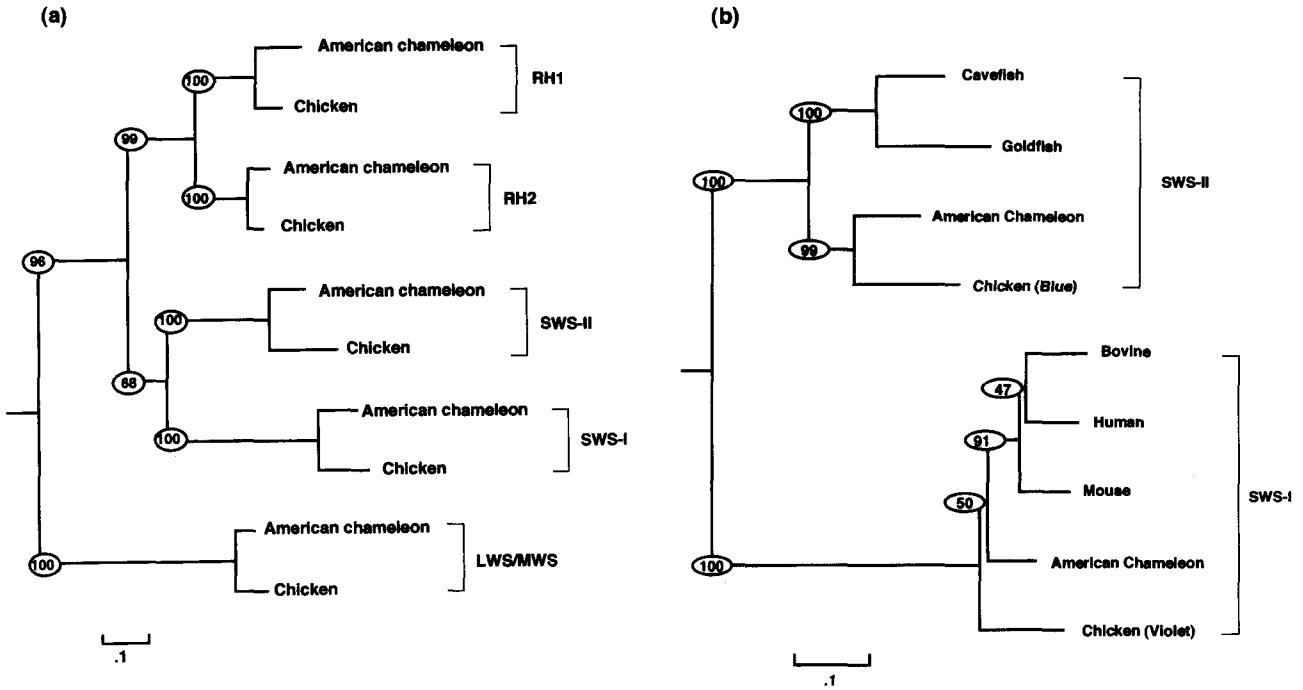

FIGURE 4. The rooted phylogenetic trees for (a) the opsins from the American chameleon and chicken and (b) the vertebrate SWS opsins reconstructed by the NJ method based on *K* values. Circled numbers indicate clustering percent supports generated by bootstrap resampling. RH1, RH2, SWS-II, SWS-I, and LWS/MWS opsins of chicken correspond to chicken rhodopsin, green, blue, violet, and red in Okano *et al.* (1992). Other SWS opsins include human blue (Nathans *et al.*, 1986), bovine and mouse blue (Chiu *et al.*, 1994), goldfish blue (Johnson *et al.*, 1993) and cavefish AF23 (Yokoyama & Yokoyama, 1993) opsins.

two loci. Since there are no frameshift mutations or premature stop codons, these genes appear to be functional. Thus, in the following, the two genes will be represented simply as $swsl_{Ac}$ using the $swsl_{Ac}$-*L* sequence.

The SWS-II gene, $sws2_{Ac}$, spans 5.5 kb from start codon to stop codon, containing five putative exons and four introns [Fig. 1(b)]. When hybridized to exon 4 of $sws2_{Ac}$, the genomic Southern blot [Fig. 2(b)] showed one clear band in each of the two restriction digests (moderately stringent conditions). The sizes of the hybridizing bands are consistent with the restriction map of the clone λBL184 in Fig. 1(b). One faint band in the *Eco*RI lane in Fig. 2(b) corresponds to the RH2 gene. Using the low stringency condition, bands corresponding to SWS-I, RH1, and RH2 genes have been detected (result not shown). These observations strongly suggest that the American chameleon genome contains only a single copy of SWS-II gene.

In all of the three genes characterized, introns are located at the identical positions as those of RH1, RH2, and SWS genes characterized to date. Splice junction signals (GT/AG) are conserved in all introns and there are no nonsense mutations in the coding regions [Fig. 3(a) and (b)].

The opsins deduced from $swsl_{Ac}$ [Fig. 3(a)] and from $sws2_{Ac}$ [Fig. 3(b)], are 347 and 365 residues long, respectively. Functionally important residues in these opsins have been conserved. They include the site of the Schiff base linkage to the chromophore (K291 in SWS-I opsin; K307 in SWS-II opsin) (Wang *et al.*, 1980), a possible *N*-glycosylation site common in SWS opsins (N12; N28) (Nathans *et al.*, 1986; Okano *et al.*, 1992; Johnson *et al.*, 1993; Yokoyama & Yokoyama, 1993; Chiu *et al.*, 1994), sites for disulfide bond (C105 and C182; C121 and C198) (Karnik *et al.*, 1988), the Schiff base counter ion (E108; E124) (Sakmar *et al.*, 1989; Zhukovsky & Oprian, 1989; Nathans, 1990), sites involved in the interaction with transducin (E129 and R130; E145 and R146) (Franke *et al.*, 1990), possible sites for the retinal binding pocket (W121, Y260, and Y263; W137, W276, and Y279) (Nakayama & Khorana, 1991), and a site involved in phosphorylation of the C-terminal region (C135; C151) (Karnik *et al.*, 1993). Multiple serines, potential targets of the opsin kinase (Ohguro *et al.*, 1994), are also observed in the C-terminal region. Thus, possessing no apparent structural defect and conservation of functionally important codons, both SWS-I and SWS-II genes in the American chameleon seem to be functional.

Phylogenetic tree of opsins from the American chameleon and chicken

We have characterized all opsin genes of the American chameleon which belong to the four major groups. The amino acid sequences of the corresponding opsins of chicken are also known (Okano *et al.*, 1992). In Fig. 4(a), the rooted phylogenetic tree for the opsins from the two

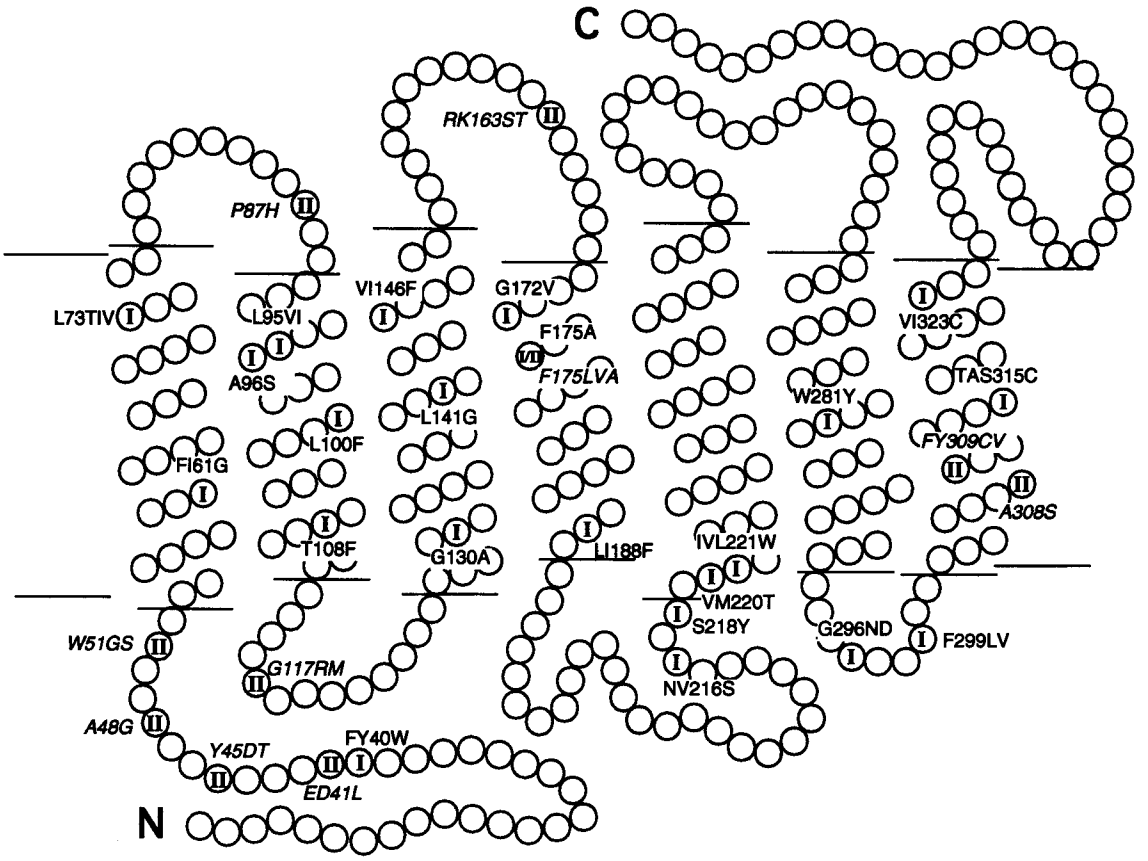

FIGURE 5. Amino acid replacements unique to SWS-I group and to SWS-II group (in italics) indicated on the TM model. Replacement sites are indicated by "I" and "II" for SWS-I- and SWS-II-specific changes, respectively. Ancestral and derived amino acids are denoted to the left and the right sides of the residue numbers, respectively. For example, RK163ST stands for an amino acid change from R or K to S or T at residue 163. The human red opsin numbering system was used (Nathans *et al.*, 1986), which contains extra 21 and 5 residues when it is compared to SWS-I and SWS-II opsins of the American chameleon in Fig. 3, respectively.

species is shown. The tree topology in Fig. 4(a) is consistent with those given in Yokoyama (1994, 1995) and Hisatomi *et al.* (1994). Note that the RH1, RH2, SWS-I, SWS-II, and LWS/MWS groups, correspond respectively to the *deuterope-*, *quartope-*, *tritope-*, *quintope-*, and *protope-*gene groups in Hisatomi *et al.* (1994) and to the Rh, M2, S, M1, and L groups in Okano *et al.* (1992). The phylogenetic tree in Okano *et al.* (1992; Fig. 5) differs from that in Fig. 4(a) in one aspect. That is, the SWS-II group is more closely related to the RH1 and RH2 groups than to SWS-I group. Since the bootstrap support for the SWS-I and SWS-II groups is only 68%, the tree topology in Fig. 4(a) is not as clear-cut as it may suggest. However, when the LWS/MWS opsins are excluded from the analysis, the support of the SWS clustering becomes much higher (Yokoyama, 1995). Figure 4(a) shows that the SWS opsin group, particularly the SWS-I group, has the longest branch length among the four groups of opsins.

Phylogenetic tree of SWS opsins

To study the phylogenetic relationship and evolutionary patterns of the vertebrate SWS opsins in more detail, the SWS opsins of the American chameleon were compared to the SWS opsins from mammals, chicken, goldfish, and cavefish (see Methods). The rooted phylogenetic tree for the vertebrate SWS opsins shows that the SWS-I and SWS-II groupings are highly repeatable [Fig. 4(b)]. Clearly, SWS-II opsin of the American chameleon is most closely related to the blue opsin of chicken, while the SWS-I opsin of the American chameleon is clustered with the mammalian SWS opsins, but the bootstrap support for that clustering is only 50%. Thus, using the currently available data set, we cannot determine whether SWS-I opsin of the American chameleon is more closely related to the mammalian SWS-I opsins or to the violet opsin of chicken.

Amino acid replacements unique to SWS groups

In order to identify amino acid replacements that are unique to SWS-I and SWS-II groups, the nine SWS opsin sequences were aligned with other opsins [9 RH1, 4 RH2, and 8 LWS/MWS opsins considered in Yokoyama (1994)]. From the aligned sequences [e.g., see Fig. 1 in Yokoyama (1994)], we first isolate highly conserved residues, where almost all amino acids of RH1, RH2, and

TABLE 1. SWS group-specific amino acid replacements in the seven transmembrane (TM) regions

	Amino acid replacements						
Opsins	TM1	TM2	TM3	TM4	TM5	TM6	TM7
SWS-I	**FI61G** **L73TIV**	L95VI A96S **L100F** **T108F**	G130A **L141G** **VI146F**	**G172V** **F175A** **LI188F**	**VM220T** **IVL221W**	W281Y	**TAS315C** **VI323C**
SWS-II				**F175LVA**			A308S **FY309CV**

Amino acid replacements associated with changes of physicochemical properties are highlighted in bold. See also Fig. 5.

LWS/MWS opsins are identical. Given such residues, we can identify the SWS-I and SWS-II group-specific amino acid replacements. We also considered less conserved residues when the derived amino acids have different physicochemical properties from the ancestral ones.

Figure 5 shows a total of 32 amino acid replacements: 22 for SWS-I opsins and 10 for SWS-II opsins. Among these, 17 and three amino acid replacements occurred in the TM regions for the SWS-I and SWS-II groups, respectively (Table 1). TM regions of opsins interact with chromophores and thus may be important in determining the absorption spectra. Thirteen out of the 17 SWS-I replacements result in a change of physicochemical properties of amino acids, while the SWS-II opsins have two equivalent changes. Thus, the change in the TM region seems to be more drastic in the SWS-I group than in SWS-II group. The number of the unique replacements outside the TM regions is about the same between the two groups: five changes for the SWS-I group and seven changes for the SWS-II group. Thus, the shorter wavelength of absorption by the SWS-I group may be caused by some of these directed amino acid replacements in the TM regions.

Some of these replacements might have modified the λ_{max} values of the visual pigments. With the exception of W281Y, none of these replacements detected in the present analysis has been tested for its effect on the absorption spectrum. At residue 281, the ancestral amino acid, W, is completely conserved among all currently known opsins including invertebrate opsins. Importantly, using site-directed mutagenesis of bovine rhodopsin, it has been shown that W281Y causes a 15 nm blue-shift of λ_{max} (Nakayama & Khorana, 1991). Thus, the amino acid changes specified in Fig. 5 will become a valuable source for directing site-directed mutagenesis studies in evaluating the effects of amino acid changes on spectral tuning by visual pigments.

Possible functions of American chameleon SWS genes

The American chameleon has three classes of visual pigments in its retina which absorb light maximally at 625, 503, and 462 nm (Provencio *et al.*, 1992). Given the λ_{max} for the A2-pigment (L2), that for the A1-pigments (L1) can be roughly estimated by $L1 = (L2\text{-}250)^{0.4} \times 52.5$ (Whitmore & Bowmaker, 1989). Thus, the λ_{max} values of the A2-pigments (L2) of 625, 503, and 462 nm of the American chameleon correspond to L1 of 562, 480, and 447 nm, respectively. American chameleon has one LWS/MWS (Kawamura & Yokoyama, 1993), one RH1 (Kawamura & Yokoyama, 1994), one RH2 (Kawamura & Yokoyama, 1995), and two SWS opsin genes. As already noted, the RH1 gene is expected to be expressed exclusively in the pineal gland. The LWS/MWS and RH2 genes seem to be responsible for λ_{max} values of 625 and 503 nm, respectively, and, furthermore, currently known vertebrate visual pigments with SWS-I and SWS-II opsins have L1 values of ~420 and ~450 nm, respectively, (see Introduction). Thus, $sws2_{Ac}$ seems most likely to encode the opsin which is responsible for λ_{max} of 462 nm, which corresponds to λ_{max} of 447 nm for A1-pigments.

Accordingly, the function of sws_{1Ac} is still unknown. Interestingly, several other *Anolis* species in Puerto Rico (*A. krugi, A. cristatellus, A. pulchellus, A. gundlachi,* and *A. evermanni*) have been shown to possess the UV-sensitive visual pigments with λ_{max} of 365 nm (Fleishman *et al.*, 1993). They also possess three other visual pigment classes with λ_{max} at 565, 495, and 450 nm, which are very close to the L1 values converted from the λ_{max} values of the American chameleon. This leaves a strong possibility that $sws1_{Ac}$ is closely related to the UV-sensitive opsin gene of the *Anolis* species in Puerto Rico. The SWS-I gene in mouse [Fig. 4(b)] has also been suspected to encode the UV opsin (see Chiu *et al.*, 1994). Unfortunately, no UV sensitivity-specific amino acid change, common to the SWS-I gene in the mouse and the American chameleon, can be identified. Because of the well-characterized opsin genes in the American chameleon, molecular analyses of the opsin genes in the Puerto Rican *Anolis* species will provide important information. Namely, the comparison of the opsin genes in the related *Anolis* species will reveal the molecular mechanism(s) involved in the development of UV-sensitivity in the *Anolis* species, which seems to be achieved independently from that in zebrafish (Yokoyama, 1995).

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Acknowledgements—Comments by Ruth Yokoyama and two anonymous reviewers were greatly appreciated. This study was supported by the USPHS Grant GM-42379.